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14. ABSTRACT Traumatic brain injury (TBI) is of great concern both to the general population and to the military. There are at least two phases to brain injury: the first is the actual injury, which may result in locally damaged tissue. The second phase is the response of the body to the injury, which may last days or weeks. This second phase includes inflammation, much of which has been shown to be harmful to the injured brain. Indeed, this inflammation may be the major cause of neuronal damage that leads to many of the negative outcomes associated with brain injury. It has been shown that several cytokines and chemokines (which are key pro-inflammatory proteins) are upregulated					
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a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER 209-228-4568

## Report Title

Biochemical study of anti-inflammatory proteins vCCI and vMIP-II

### ABSTRACT

Traumatic brain injury (TBI) is of great concern both to the general population and to the military. There are at least two phases to brain injury: the first is the actual injury, which may result in locally damaged tissue. The second phase is the response of the body to the injury, which may last days or weeks. This second phase includes inflammation, much of which has been shown to be harmful to the injured brain. Indeed, this inflammation may be the major cause of neuronal damage that leads to many of the negative outcomes associated with brain injury. It has been shown that several cytokines and chemokines (which are key pro-inflammatory proteins) are upregulated upon brain injury. We have studied the structural biology and biochemistry of two proteins, vCCI and vMIP-II, each of which has been shown to have powerful anti-inflammatory properties due to inhibition of chemokines (and each having a separate mechanism of action). This work has included mutagenesis, binding studies, and structural studies by NMR. A greater understanding of these anti-inflammatory proteins can allow a molecular understanding of chemokine inhibition. This could eventually lead to strategies for recovery from traumatic brain injury.

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**Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:**

#### **(a) Papers published in peer-reviewed journals (N/A for none)**

Received

Paper

07/13/2014 4.00 Y.-G. Gao, M. S. Schill, N. Isern, N.-W. Kuo, C. M. Dupureur, P. J. LiWang. Structural insights into the interaction between a potent anti-inflammatory protein, vCCI, and the human CC chemokine, Eotaxin-1, Journal of Biological Chemistry, (01 2014): 0. doi: 10.1074/jbc.M113.538991

**TOTAL: 1**

**Number of Papers published in peer-reviewed journals:**

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#### **(b) Papers published in non-peer-reviewed journals (N/A for none)**

Received

Paper

08/29/2013 1.00 1. Jie Xue1,, 2. Nai-Wei Kuo1, , 3. Megan Schill, , 4. Patricia J. LiWang. A comparison of 5P12-vMIP-II and vMIP-II as HIV-1 entry inhibitors, Journal of Biochemistry and Physiology, (02 2013): 0. doi:

**TOTAL: 1**

Number of Papers published in non peer-reviewed journals:

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(c) Presentations	
1. Invited Seminar: "Biochemical Design of Anti-Inflammatory Proteins and HIV Entry Inhibitors" Lawrence Livermore National Labs; Livermore, CA, November 2012	
2. Invited Seminar: "Biochemical Investigation of Anti-Inflammatory Proteins and HIV Entry Inhibitors" University of California Davis; Davis, CA, Feb, 2013	
3. Poster presentation by undergraduate Megan Schill: "Combating inflammation in diseases and injury:vCCI, a potent anti-inflammatory protein" Merced, CA. October 2012.	
4. Invited Seminar: "Biochemical Design and Investigation of HIV entry inhibitors". California State University Fresno Sept, 2012	
5. Poster presentation by undergraduate Megan Schill: "Studying the Interactions between the Anti-inflammatory Protein vCCI and various CC Chemokines ". Merced, CA. March 2013	
6. Poster presentation at the International Biophysical Society meeting "Insights into the interaction between the potent anti-inflammatory protein vCCI and the chemokine Eotaxin" Feb, 2013	
7. Invited Seminar: "Biochemical Investigation of Anti-Inflammatory Proteins and HIV Entry Inhibitors" Biophysics group, University of California Davis	
Number of Presentations:	5.00

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Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Peer-Reviewed Conference Proceeding publications (other than abstracts):

<u>Received</u>	<u>Paper</u>
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08/22/2013	3.00	3. Megan S. Schill, , 4. Cynthia M. Dupureur, , 5. Patricia J. LiWang, 1. Nai-Wei Kuo, , 2. Yong-Guang Guo, . Insights into the interaction between the potent anti-inflammatory protein vCCI and the chemokine Eotaxin, Biophysical Society 57th Annual Meeting-Philadelphia, PA. 02-FEB-13, . : ,
TOTAL:	1	

(d) Manuscripts

Received

Paper

08/26/2013 2.00 1. Nai-Wei Kuo,, 2. Yong-Guang Gao,, 3. Megan Schill1, , 4. Nancy Isern, , 5. Cynthia M. Dupureur, , 6. Patricia J. LiWang. Structural insights into the interaction between a potent anti-inflammatory protein, vCCI, and human CC chemokine, Eotaxin, (  
)

TOTAL: 1

Number of Manuscripts:

Books

Received

Book

TOTAL:

Received

Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

## Awards

Undergraduates:

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Megan Schill (URAP recipient): Grossman award for excellence in academics and research winner, Spring 2013. Only one student among all chemistry majors (about 180 students) was chosen for this award in 2013.

Christopher Fisher, undergrad with funding from the Army Undergraduate Research Apprenticeship Program (URAP): Received the Navy HPSP Medical Corps scholarship, which will fully fund his medical school and requires him to join the Navy afterward. He will start medical school in the Fall of 2014. This student won the Grossman Award for outstanding undergraduate in Biological Sciences (2 given for 1000 students) This student also was on the Dean's list and Chancellor's Honor list in 2012 and 2013.

Craig Fisher, also a URAP recipient: Deans and Chancellor's list in 2012 and 2013, Robert C. Byrd Scholarship recipient, chosen for selective FBI internship summer 2013.

Mike Jian: School of Natural Sciences Outstanding Undergraduate Spring 2014. (Only 2 awards given for about 1500 students in all majors of the School of Nat Sci). Grossman Scholarship 2012 for outstanding lower division chemistry student. Dean's list every semester.

Hawi Gameda (new student in 2014) Admitted to the selective UC LEADS program for under-rep students; Chancellor's list 2013; Benton Honors scholarship 2013; Westly scholarship 2014.

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### Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
<b>FTE Equivalent:</b>	
<b>Total Number:</b>	

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### Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
Li Zhang	1.00
Nicole Kuo	1.00
Dr. Yong-guang Gao	1.00
<b>FTE Equivalent:</b>	<b>3.00</b>
<b>Total Number:</b>	<b>3</b>

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### Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
Patricia LiWang	0.10	
<b>FTE Equivalent:</b>	<b>0.10</b>	
<b>Total Number:</b>	<b>1</b>	

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### Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Megan Schill	0.50	Chemistry; supported for summers,during academic ye
Craig Fisher	0.25	Biosciences
Christopher Fisher	0.25	Biosciences
Hawi Gameda	0.10	Biosciences; new student continues project
Mike Jian	0.10	Chemistry; vMIP project but no salary
<b>FTE Equivalent:</b>	<b>1.20</b>	
<b>Total Number:</b>	<b>5</b>	

### Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: ..... 3.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 3.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 3.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 3.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense ..... 2.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: ..... 2.00

### Names of Personnel receiving masters degrees

NAME

**Total Number:**

### Names of personnel receiving PHDs

NAME

**Total Number:**

### Names of other research staff

NAME

PERCENT SUPPORTED

**FTE Equivalent:**

**Total Number:**

### Sub Contractors (DD882)

### Inventions (DD882)

### Scientific Progress

Please see attached PDF for research summary, future plans, paragraph on undergraduate education, and papers published.

### Technology Transfer

## Scientific Progress and Accomplishments

Statement of Problem Studied: Traumatic Brain Injury (TBI) is a great ongoing concern for both the military and the general public. In recent years, it has become clear that inflammation following the initial injury is a major cause of the long-term effects of TBI. Our lab studies the basic biochemistry two potent anti-inflammatory proteins (vCCI and vMIP-II) that could eventually be used in the treatment or to allow better understanding of TBI. vCCI is a protein that binds chemokines, which are pro-inflammatory proteins. vMIP-II is a chemokine analog that binds chemokine receptors and blocks other chemokines from acting on those receptors.

We have successfully completed most of the Aims of the proposal. Much of the work for Aim 1 was recently published (Kuo, Gao et al. 2014).

Specific Aim 1: Determine how vCCI is able to tightly bind CC chemokines using a combination of mutagenesis, biophysical, and structural techniques

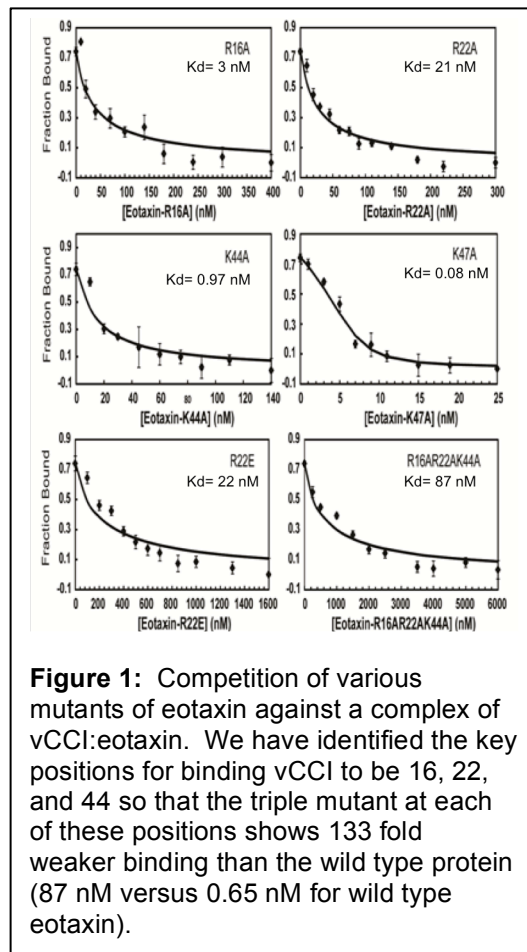
For Aim 1: “Probing the vCCI:chemokine complex: Mutations in the chemokine”, we have produced and purified all the desired chemokine variants, and tested them in triplicate for binding to vCCI. In this aim, our goal has been to determine which chemokine residues are critical for binding vCCI. Our approach has been to make mutations in various pro-inflammatory chemokines (particularly the well-behaved chemokine eotaxin) and to have these chemokines compete for binding to vCCI with a chemokine having a fluorescent probe (eotaxin-fluor). The variants are thereby tested for ability to bind vCCI so that we can determine which amino acids are critical for vCCI binding. Results are shown in Table 1.

**Table 1:** Fluorescence anisotropy results of the interaction between vCCI and eotaxin mutants or other CC chemokines.

Mutants	K <sub>d</sub> , nM	fold change <sup>a</sup> (K <sub>d</sub> <sub>mut</sub> /K <sub>d</sub> <sub>wt</sub> )
wt eotaxin	0.650 ± 0.170	1.00
F11A	1.67 ± 0.47	2.57
R16A	3.13 ± 0.61	4.82
R22A	1.29 ± 0.57	1.98
R22E	21.5 ± 2.5	33.1
K44A	0.970 ± 0.340	1.49
K47A	0.0800 ± 0.0600	0.120
R16AR22A	11.8 ± 2.2	18.2
R22AK44A	3.09 ± 0.93	4.75
R16AR22E	27.0 ± 8.0	41.5
R16AR22AK44A	86.5 ± 28.5	133
MCP-1	1.09 ± 0.11	1.68
MIP-1β	1.16 ± 0.17	1.78
MIP-1β-K45AR46AK48A	2.18 ± 0.36	3.35
RANTES	0.220 ± 0.090	0.340

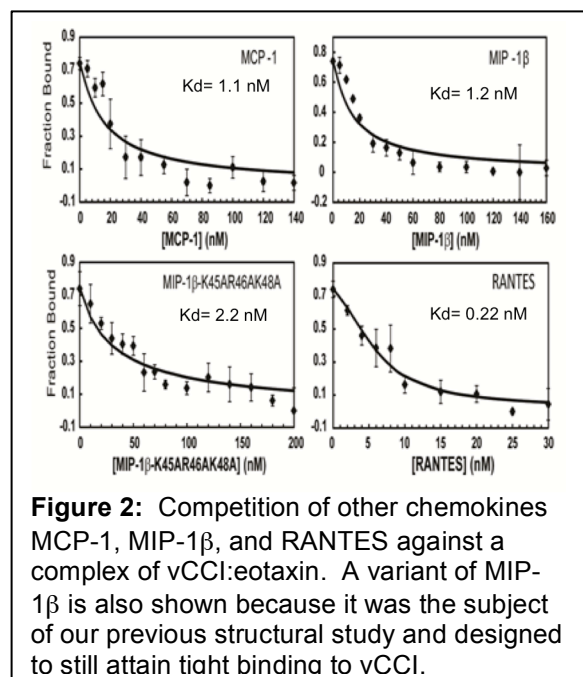
<sup>a</sup> fold change indicates change in K<sub>d</sub> compared to wild type eotaxin.

To provide more explanation of the experiments that produced Table 1, Figure 1 shows the competition fluorescence assay that was used. In this assay, a vCCI:eotaxin-fluor complex is first made. Then mutant chemokine is titrated into the tube. The mutant chemokine will compete off the eotaxin-fluor, causing a drop in the fluorescence anisotropy signal. This curve can be used to determine the binding constant of the mutant, and Figure 1 shows a sample of these competition curves. Our conclusion from the data in Table 1 and Figure 1 is that the negatively charged surface of vCCI allows it to bind to several key positively charged residues in CC chemokines. In particular, the positive charges at the 16<sup>th</sup>, 22<sup>nd</sup>, and 44<sup>th</sup> position (using eotaxin numbering) appear to be important in vCCI binding. CC chemokines in general tend to have positive charges in some or all of these positions. Interestingly, and providing an explanation for how vCCI is able to broadly bind dozens of chemokines (rather than just tightly and selectively bind a few) we have observed that any single point mutant in these positions in the chemokine only results in a few fold reduction in ability to bind vCCI. However, when all three positions are mutated (eotaxin R16AR22AK44A), a 133-fold loss of binding ability to vCCI is observed.



**Figure 1:** Competition of various mutants of eotaxin against a complex of vCCI:eotaxin. We have identified the key positions for binding vCCI to be 16, 22, and 44 so that the triple mutant at each of these positions shows 133 fold weaker binding than the wild type protein (87 nM versus 0.65 nM for wild type eotaxin).

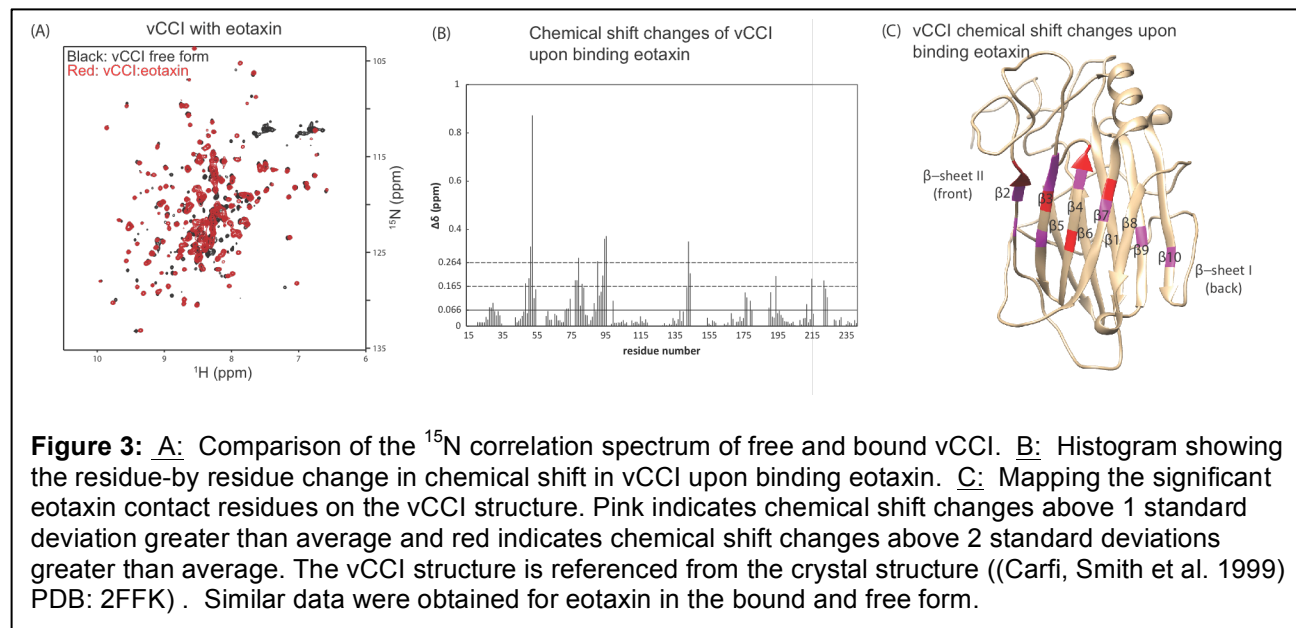
We have also obtained results with other chemokines, as they compete for binding to vCCI. As shown in Figure 2, pro-inflammatory chemokines MCP-1, MIP-1 $\beta$  and RANTES all are shown to bind to vCCI, with RANTES (the most basic of these) binding vCCI the most tightly. In our recently published paper (Kuo et al, 2014) we compare the sequence of these chemokines and show that, as our mutational studies have shown, the chemokines with positive charges at the three key positions (16, 22, 44 in eotaxin numbering) show tight binding to vCCI.



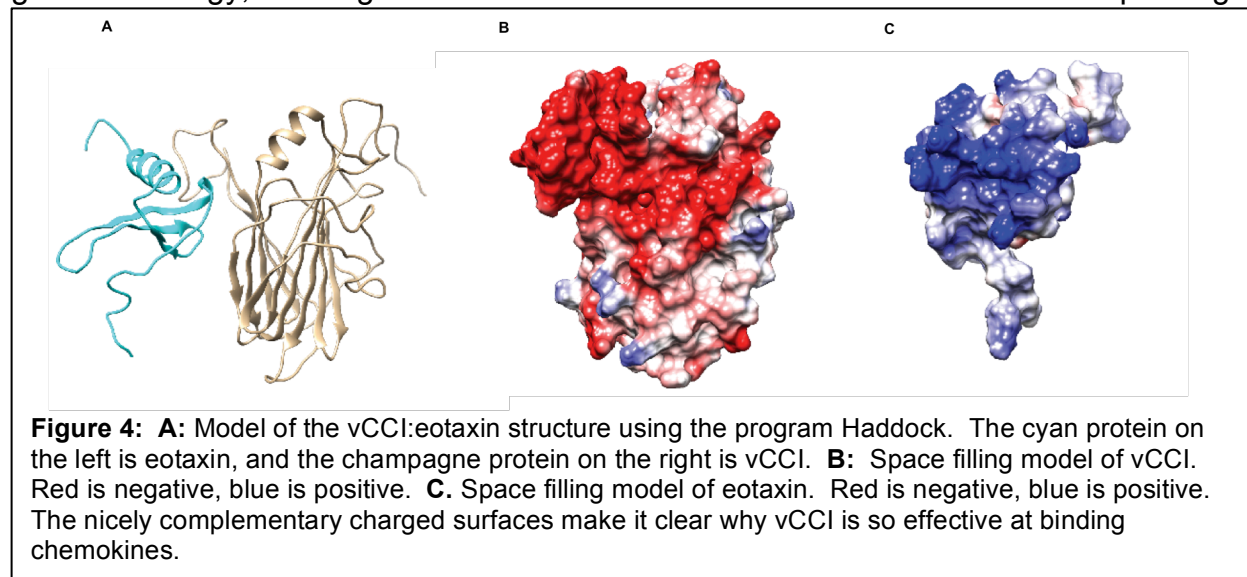
**Figure 2:** Competition of other chemokines MCP-1, MIP-1 $\beta$ , and RANTES against a complex of vCCI:eotaxin. A variant of MIP-1 $\beta$  is also shown because it was the subject of our previous structural study and designed to still attain tight binding to vCCI.

For Aim 1, “Structural studies of vCCI in complex with pro-inflammatory chemokines”, multidimensional NMR studies were undertaken with the goal being to obtain structural data on the complex between vCCI and CC chemokines. We have obtained chemical shift assignments of the free and bound form of both vCCI and the chemokine Eotaxin. These assignments (in which the amide group from each amino acid is identified on the spectrum) allow a comparison of the bound and unbound form, which can then be used to

make a model of the vCCI:eotaxin complex. These results require the interpretation of many different NMR experiments, measured on variously isotopically labeled NMR samples. Figure 3 shows an overlay of the spectra of free and bound vCCI and a residue-by-residue measurement of the chemical shift change between the bound and unbound for. Figure 3C maps these changes onto the structure of vCCI, showing that chemokine binding is likely along one face of the protein.



Our structural work has allowed us to obtain a model of the vCCI:eotaxin complex, shown in Figure 4. Similar to our mutagenesis results, the overall data indicate that vCCI uses a negatively charged surface to bind positive charges on the chemokine. This is likely a general strategy, allowing vCCI to bind numerous different chemokines and explaining its



broad anti-inflammatory effects.

For the last portion of Aim 1, which is “Probing the vCCI:chemokine complex: Mutations in vCCI”, we believe that mutations in vCCI are not necessary at this time, since a

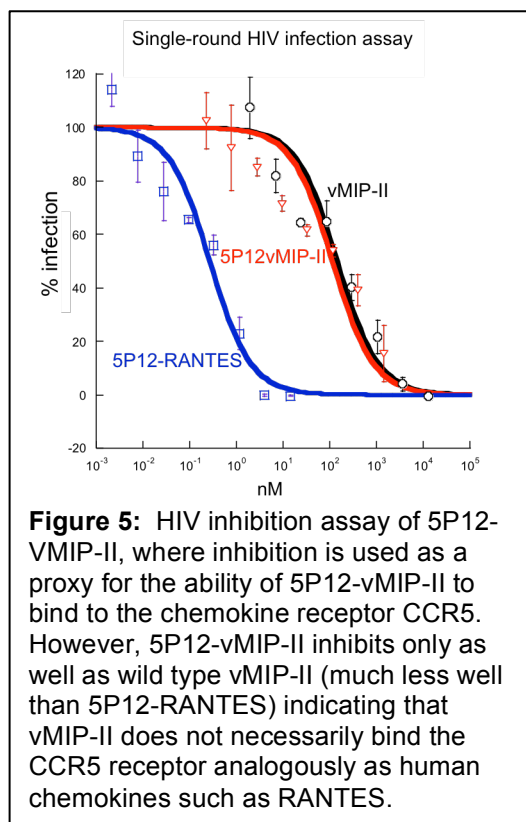
group has recently published several of the mutations that we had planned to make (White et al., 2011). Rather, we hope to respond to future Requests for Proposals by moving our work in the direction of using our results to start work in cell culture, including attempting to reduce inflammatory markers in neuronal cells.

Specific Aim 2: Determine the biochemical basis for the ability of vMIP-II (a chemokine analog) to interfere with the chemokine system by studying its ability to bind the chemokine receptor CCR5.

Our work with Aim 2 is progressing well: One part of Aim 2 is to “replace the N-terminus of vMIP-II with a known receptor-affinity segment to increase the potency of its anti-inflammatory function”. The goal is to use the results of a study from the Hartley group (Gaertner, Cerini et al. 2008) where it was shown that the chemokine RANTES could be engineered to have higher affinity for its receptor. The hypothesis is that if vMIP-II binds CCR5 similarly to human chemokines then if these amino acids are placed into vMIP-II, it also will have higher affinity for this receptor, allowing it to be an even better chemokine inhibitor.

We have successfully mutated vMIP-II to have the proposed amino acids in place and have produced and purified this variant (“5P12-vMIP-II”). As a way to determine the ability of this protein to bind the CCR5 receptor, we took advantage of the fact that the CCR5 receptor is used as a portal by the HIV virus to enter human cells. So initial assays were carried out to determine if 5P12-vMIP-II was an effective HIV inhibitor as a proxy for ability to bind CCR5. This work has resulted in a publication (due to the high interest in the HIV community for potential tight binding HIV inhibitors; (Xue, Kuo et al. 2013). One of our Army URAP undergraduates, Megan Schill, did a significant amount of work on this project and is an author on the paper.

As shown in Figure 5, 5P12-vMIP-II does not inhibit HIV better than the wild type protein, indicating that the change at the N-terminus did not improve its ability to bind CCR5. This suggests that vMIP-II does not bind the CCR5 receptor in the same way as natural human chemokines such as RANTES. This provides evidence for our evolving hypothesis that vMIP-II is able to bind so many different CC chemokine receptors due to its similar tertiary structure and abundance of positive charges on its binding surface. The ability to be too specific in binding one chemokine receptor would lead to lack of ability to bind others. This work with vMIP-II (a chemokine analog) relates to our work with vCCI (a chemokine binding protein), where we showed that vCCI is able to bind so many different chemokines due to its general negatively charged surface, allowing it to bind to chemokines that have positive charges in a few key locations. In both cases, charge complementarity seems to be a key point in these important inflammatory actions, with



positive charges on the chemokine (or chemokine homolog) interacting with negative charges on the receptor (or chemokine binding protein).

The other part of Aim 2 is to investigate the role of the amino acid Phe 13 in vMIP-II in binding to chemokine receptors. This residue is very important for other chemokines in binding receptors, but it appears that vMIP-II can bind chemokine receptors even without a Phe at position 13. As part of the larger goal to understand how vMIP-II can bind and block numerous chemokine receptors, while possibly using a strategy that is not the same as other chemokines, we have mutated position 13 in order to determine the ability of the variants to bind receptor CCR5. The genes for several vMIP-II variants have been made by undergraduates in the lab and put into a SUMO expression system for easy purification.

However, this is one area of research that has stopped before experiments could be complete. Our plan was to determine affinity to CCR5 in cell-based assays using the “scintillation proximity assay” (SPA) that utilizes a small amount of radiolabeled chemokine. The labeled chemokine will cause a scintillant-bead to emit light that can be detected with a luminescence plate reader, and our variants can compete with that process. We started these assays, but were not successful. While our plate reader should have been able to detect photons from the scintillant, it was not sufficiently sensitive to produce competition curves. Even after sending the luminometer to the manufacturer, we were not successful in getting enough sensitivity for the SPA assay to produce results. (It was suggested that we invest in a newer, better plate reader, which will have to wait for a future equipment grant.)

**Future Plans:** We are very appreciative of the Army funding, and plan to continue work on anti-inflammatory strategies in two ways. First, we would like to continue this work by studying vCCI in complex with the chemokine analog vMIP-II, thereby observing a complex between a highly evolved chemokine binding protein with a highly evolved chemokine analog. We have measured NMR spectra of this complex, and it is of high enough quality for a structure determination. We have also carried out quantitative binding studies by fluorescence and observe that vMIP-II binds to vCCI better than any other chemokine that we have studied, making it a picomolar binding partner.

Our second goal for the future is to study the effect of our anti-inflammatory proteins on cell culture containing neuronal cells and astrocytes. These experiments would provide preliminary results suggesting whether our proteins are protective of actual brain-derived cells, such as those injured in TBI. It is our hope to apply to future Army calls for proposals, if any should be announced.

### **Summary of results with relevance to the Army:**

#### **Aim 1:**

vCCI is a powerful anti-inflammatory protein that could be used in the treatment or study of traumatic brain injury.

- We have made good progress in understanding the mechanism of this protein, which functions by binding and inhibiting chemokines.
- We have shown specific amino acids in the chemokine that are important in the vCCI-chemokine interaction. These include positively charged amino acids at positions 16, 22, 44

(using eotaxin numbering).

- Our results suggest that vCCI maintains broad spectrum ability to bind chemokines by interacting favorably if chemokines retain some or most of the positive charges in those key positions, allowing variation in chemokine sequence.
- We have used multidimensional NMR to obtain chemical shift assignments and to identify which amino acids are likely involved in the vCCI:chemokine interface. This has provided data to obtain a published, structural model of the vCCI-eotaxin complex.

#### Aim 2:

vMIP-II is a chemokine homolog that has anti-inflammatory properties (because it mimics chemokines without being able in general to attract inflammatory cells) that could be used in the treatment or study of traumatic brain injury.

- We have produced and purified 5P12-VMIP-II, a vMIP-II variant that was hypothesized to bind the chemokine receptor CCR5 (which is necessary for immune activation by some chemokines) more tightly than wild type vMIP-II.
- We have found that 5P12-vMIP-II likely does not bind the CCR5 receptor in the same way as human chemokines. This may allow it to bind a wide variety of receptors, explaining its broad anti-inflammatory capability.

**Education of students:** This grant has funded 3 undergraduates with direct research stipends, and several other undergrads through research experience. One of the stipend-supported students has graduated and is currently enlisted in the Navy (in a Navy-supported medical program), and another plans enlistment in the Navy. The third stipend-supported student is a woman majoring in Chemistry, who has won an academic award. Other students in my laboratory include two African American women (one current, one in the past year) as well as other bright students from a mixture of backgrounds.

#### **Publications from this proposal:**

1. "A comparison of 5P12-vMIP-II and vMIP-II as HIV-1 entry inhibitors"  
Jie Xue, Nai-Wei Kuo, Megan Schill, and Patricia J. LiWang *Biochemistry and Physiology* (2013) S2. doi:10.4172/2168-9652.S2-005.
2. "Structural insights into the interaction between a potent anti-inflammatory protein, viral CC chemokine Inhibitor (vCCI), and human CC chemokine, Eotaxin-1"  
Nai-Wei Kuo Yong-Guang Gao, Megan Schill\*, Nancy Isern, Cynthia M. Dupureur, and Patricia J. LiWang *J. Biol. Chem* 289, 6592-7703 (2014).
3. "Insights into the interaction between the potent anti-inflammatory protein vCCI and the chemokine Eotaxin"  
Nai-Wei Kuo, Yong-Guang Guo, Megan S. Schill\*, Cynthia M. Dupureur, and Patricia J. LiWang  
Presentation and Abstract in the Proceedings of the 57th Biophysical Society annual meeting, Feb. 2013, Philadelphia, PA.

- *Megan Schill is an ARO URAP undergraduate student*

**Scientific Presentations from this Proposal:** See appropriate section from Progress Report web site.

**Brief bibliography of Progress Report** (see above for publications from our group from this proposal):

Carfi, A., C. A. Smith, P. J. Smolak, J. McGrew and D. C. Wiley (1999). "Structure of a soluble secreted chemokine inhibitor vCCI (p35) from cowpox virus." Proc. Natl. Acad. Sci. USA **96**: 12379-12383.

Gaertner, H., F. Cerini, J. M. Escola, G. Kuenzi, A. Melotti, R. Offord, I. Rossitto-Borlat, R. Nedellec, J. Salkowitz, G. Gorochoy, D. Mosier and O. Hartley (2008). "Highly potent, fully recombinant anti-HIV chemokines: reengineering a low-cost microbicide." Proc Natl Acad Sci U S A **105**(46): 17706-17711.

Kuo, N. W., Y. G. Gao, M. S. Schill, N. Isern, C. M. Dupureur and P. J. Liwang (2014). "Structural insights into the interaction between a potent anti-inflammatory protein, viral CC chemokine inhibitor (vCCI), and the human CC chemokine, Eotaxin-1." J Biol Chem **289**(10): 6592-6603.

Xue, J., N.-W. Kuo, M. Schill and P. J. LiWang (2013). "A comparison of 5P12-vMIP-II and vMIP-II as HIV-1 entry inhibitors." Biochemistry and Physiology.